

Supplementary Material

Codon usage is a defining parameter for the allotopic expression of mtDNA genes

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Supplementary Table S1

Amino acid and codon frequencies in the human nuclear genome and in allotopically expressed mitochondrial genes.

AA/Codon	Nuclear %		Recoded %		Optimized %	
ALA	6.93		6.73		6.73	
GCU	1.84		1.35		0.98	
GCC	2.77		3.09		4.49	
GCA	1.58		2.09		1.27	
GCG	0.74		0.21		0.0	
ARG	5.67		1.66		1.66	
CGU	0.45		0.18		0	
CGC	1.04		0.63		0.26	
CGA	0.62		0.66		0.03	
CGG	1.14		0.11		0.53	
AGA	1.22		0.05		0.45	
AGG	1.2		0.03		0.4	
ASN	3.61		4.33		4.33	
AAU	1.7		0.92		1.98	
AAC	1.91		3.41		2.35	
ASP	4.69		1.74		1.74	
GAU	2.18		0.50		0.74	
GAC	2.51		1.24		1.00	
CYS	2.32		0.58		0.58	
UGU	1.06		0.16		1.13	
UGC	1.26		0.42		0.45	
GLN	4.65		2.38		2.38	
CAA	2.9		1.95		0.03	
CAG	3.42		0.42		2.35	
GLU	6.86		2.32		2.32	
GAA	2.9		1.64		0.40	
GGG	3.96		0.69		1.93	
GLY	6.6		5.60		5.60	
GGU	1.08		0.63		0.0	
GGC	2.22		2.24		3.56	
GGA	1.65		1.72		1.56	
GGG	1.65		1.00		0.48	
HIS	2.6		2.56		2.56	
CAU	1.09		0.55		0.32	
CAC	1.51		2.01		2.24	
ILE	4.43		8.42		8.42	
AUU	1.6		3.46		0.92	
AUC	2.08		4.96		7.50	
AUA	0.75		0.00		0.00	

LEU		10.02		17.00		17.00
	UUA		0.77		1.74	0.00
	UUG		1.29		0.42	0.00
	CUU		1.32		1.53	0.00
	CUC		1.96		3.85	0.00
	CUA		0.72		6.63	0.00
	CUG		3.96		2.82	17.00
LYS		5.63		2.51		2.51
	AAA		2.44		2.09	0.34
	AAG		3.19		0.42	2.16
MET		2.2		5.46		5.49
	AUG		2.2		5.46	5.49
PHE		3.79		5.70		5.70
	UUU		1.76		2.11	2.59
	UUC		2.03		3.59	3.11
PRO		6.11		5.78		5.78
	CCU		1.75		1.19	1.98
	CCC		1.98		3.09	1.90
	CCA		1.69		1.35	1.90
	CCG		0.69		0.16	0.00
SER		8.11		7.23		7.23
	UCU		1.52		1.00	1.90
	UCC		1.77		2.51	2.32
	UCA		1.22		1.93	0.29
	UCG		0.44		0.18	0.00
	AGU		1.21		0.48	0.29
	AGC		1.95		1.14	2.43
THR		5.32		9.27		9.26
	ACU		1.31		1.48	0.77
	ACC		1.89		4.09	4.65
	ACA		1.51		3.46	3.85
	ACG		0.61		0.24	0.00
TRP		1.32		2.75		2.74
	UGG		1.32		2.75	2.74
TYR		2.75		3.56		3.56
	UAU		1.22		1.24	1.50
	UAC		1.53		2.32	2.06
VAL		5.44		4.41		4.41
	GUU		1.1		0.79	0.00
	GUC		1.45		1.29	0.58
	GUA		0.71		1.69	0.00
	GUG		2.18		0.63	3.83

Supplementary Tables S2

RSCU values for nuclear genes and for minimally-recoded and codon-optimized mitochondrial genes

Nuclear Genes															
CODON	AA	Freq/AA	RSCU	CODON	AA	Freq/AA	RSCU	CODON	AA	Freq/AA	RSCU	CODON	AA	Freq/AA	RSCU
UUU	F	0.46	0.92	UCU	S	0.19	1.14	UAU	Y	0.44	0.88	UGU	C	0.46	0.92
UUC	F	0.54	1.08	UCC	S	0.22	1.32	UAC	Y	0.56	1.12	UGC	C	0.54	1.08
UUA	L	0.08	0.48	UCA	S	0.15	0.90	UAA	*	0.30	0.90	UGA	*	0.47	1.41
UUG	L	0.13	0.78	UCG	S	0.05	0.30	UAG	*	0.24	0.72	UGG	W	1.00	1.00
CUU	L	0.13	0.78	CCU	P	0.29	1.16	CAU	H	0.42	0.84	CGU	R	0.08	0.48
CUC	L	0.20	1.20	CCC	P	0.32	1.28	CAC	H	0.58	1.16	CGC	R	0.18	1.08
CUA	L	0.07	0.42	CCA	P	0.28	1.12	CAA	Q	0.27	0.54	CGA	R	0.11	0.66
CUG	L	0.40	2.40	CCG	P	0.11	0.44	CAG	Q	0.73	1.46	CGG	R	0.20	1.20
AUU	I	0.36	1.08	ACU	T	0.25	1.00	AAU	N	0.47	0.94	AGU	S	0.15	0.90
AUC	I	0.47	1.41	ACC	T	0.36	1.44	AAC	N	0.53	1.06	AGC	S	0.24	1.44
AUA	I	0.17	0.51	ACA	T	0.28	1.12	AAA	K	0.43	0.86	AGA	R	0.21	1.26
AUG	M	1.00	1.00	ACG	T	0.11	0.44	AAG	K	0.57	1.14	AGG	R	0.21	1.26
GUU	V	0.18	0.72	GCU	A	0.27	1.08	GAU	D	0.46	0.92	GGU	G	0.16	0.64
GUC	V	0.24	0.96	GCC	A	0.40	1.60	GAC	D	0.54	1.08	GGC	G	0.34	1.36
GUA	V	0.12	0.48	GCA	A	0.23	0.92	GAA	E	0.42	0.84	GGA	G	0.25	1.00
GUG	V	0.46	1.84	GCG	A	0.11	0.44	GAG	E	0.58	1.16	GGG	G	0.25	1.00

Codon-Optimized Mitochondrial Genes															
CODON	AA	Freq/AA	RSCU	CODON	AA	Freq/AA	RSCU	CODON	AA	Freq/AA	RSCU	CODON	AA	Freq/AA	RSCU
UUU	F	0.45	0.91	UCU	S	0.26	1.58	UAU	Y	0.42	0.84	UGU	C	0.23	0.45
UUC	F	0.55	1.09	UCC	S	0.32	1.93	UAC	Y	0.58	1.16	UGC	C	0.77	1.55
UUA	L	0.00	0.00	UCA	S	0.04	0.24	UAA	*	--	--	UGA	*	--	--
UUG	L	0.00	0.00	UCG	S	0.00	0.00	UAG	*	--	--	UGG	W	1.00	1.00
CUU	L	0.00	0.00	CCU	P	0.34	1.37	CAU	H	0.12	0.25	CGU	R	0.00	0.00
CUC	L	0.00	0.00	CCC	P	0.33	1.32	CAC	H	0.88	1.75	CGC	R	0.16	0.95
CUA	L	0.00	0.00	CCA	P	0.33	1.32	CAA	Q	0.01	0.02	CGA	R	0.02	0.10
CUG	L	1.00	6.00	CCG	P	0.00	0.00	CAG	Q	0.99	1.98	CGG	R	0.32	1.90
AUU	I	0.11	0.33	ACU	T	0.08	0.33	AAU	N	0.46	0.91	AGU	S	0.04	0.24
AUC	I	0.89	2.67	ACC	T	0.50	2.01	AAC	N	0.54	1.09	AGC	S	0.34	2.01
AUA	I	0.00	0.00	ACA	T	0.42	1.66	AAA	K	0.14	0.27	AGA	R	0.27	1.62
AUG	M	1.00	1.00	ACG	T	0.00	0.00	AAG	K	0.86	1.73	AGG	R	0.24	1.43
GUU	V	0.01	0.02	GCU	A	0.15	0.58	GAU	D	0.42	0.85	GGU	G	0.00	0.00
GUC	V	0.13	0.53	GCC	A	0.67	2.67	GAC	D	0.58	1.15	GGC	G	0.64	2.55
GUA	V	0.00	0.00	GCA	A	0.19	0.75	GAA	E	0.17	0.34	GGA	G	0.28	1.11
GUG	V	0.87	3.47	GCG	A	0.00	0.00	GAG	E	0.83	1.66	GGG	G	0.08	0.34

Minimally-Recorded Mitochondrial Genes

CODON	AA	Freq/AA	RSCU												
UUU	F	0.36	0.71	UCU	S	0.12	0.70	UAU	Y	0.34	0.68	UGU	C	0.23	0.45
UUC	F	0.64	1.29	UCC	S	0.36	2.17	UAC	Y	0.66	1.32	UGC	C	0.77	1.55
UUA	L	0.11	0.68	UCA	S	0.30	1.82	UAA	*	--	--	UGA	*	--	--
UUG	L	0.03	0.17	UCG	S	0.03	0.15	UAG	*	--	--	UGG	W	1.00	1.00
CUU	L	0.10	0.61	CCU	P	0.19	0.75	CAU	H	0.19	0.37	CGU	R	0.11	0.67
CUC	L	0.26	1.56	CCC	P	0.54	2.17	CAC	H	0.81	1.63	CGC	R	0.41	2.48
CUA	L	0.43	2.57	CCA	P	0.24	0.95	CAA	Q	0.91	1.82	CGA	R	0.44	2.67
CUG	L	0.07	0.42	CCG	P	0.03	0.13	CAG	Q	0.09	0.18	CGG	R	0.03	0.19
AUU	I	0.39	1.16	ACU	T	0.15	0.59	AAU	N	0.20	0.39	AGU	S	0.05	0.31
AUC	I	0.61	1.84	ACC	T	0.44	1.77	AAC	N	0.80	1.61	AGC	S	0.14	0.85
AUA	I	0.00	0.00	ACA	T	0.38	1.53	AAA	K	0.89	1.79	AGA	R	0.03	0.19
AUG	M	1.00	1.00	ACG	T	0.03	0.11	AAG	K	0.11	0.21	AGG	R	0.02	0.10
GUU	V	0.19	0.74	GCU	A	0.17	0.67	GAU	D	0.23	0.45	GGU	G	0.11	0.45
GUC	V	0.29	1.15	GCC	A	0.49	1.95	GAC	D	0.77	1.55	GGC	G	0.41	1.64
GUA	V	0.42	1.68	GCA	A	0.31	1.25	GAA	E	0.73	1.45	GGA	G	0.32	1.26
GUG	V	0.11	0.43	GCG	A	0.03	0.13	GAG	E	0.27	0.55	GGG	G	0.16	0.64

Supplementary Table S3

ARMS qPCR Primers ND1^m and A8/A6^{mut}

ND1^m (m.3571dupC)	Wild type	Mutant
ND1WT-F	5' TCTACTATGAACCCCCAT3'	5' TCTACTATGAACCCCCCGT3'
ND1Mut-F	5' TCTACTATGAACCCCCATC3'	5' TCTACTATGAACCCCCCGTC3'
ND1-R	5' GTTTGATGCTCACCCCTGA3'	
A8/A6^{mut} (m.8529G>A)	Wild type	Mutant
ATP8WT-F	5' TTATAACAAACCCTGAGAACCA AAACG3'	5' TTATAACAAACCCTGAGAACCA AAACA3'
ATP8Mut-F	5' TTATAACAAACCCTGAGAACCA AAATG3'	5' TTATAACAAACCCTGAGAACCA AAATA3'
ATP8-R	5' GTACTGATCATTCTATTTCC3'	
Control Genes	Forward Primer	Reverse Primer
<i>MT-CYB</i>	5' CTAACCCCTAATAAAATTAAT TAA3'	5' GTCTGGTGAGTAGTGCATGG3'
<i>MT-COX3</i>	5' AGTACTTCGAGTCTCCC3'	5' GACGTGAAGTCCGTGG3'

Supplementary Table S4

Homoplasmy Confirmation using ARMS qPCR for m.3571insC (ND1^m)

Input (gBlock gene copy #)	Mean $\Delta\Delta C_T$ Mut-WT	Input (143B WT/ND1 ^m mtDNA)	Mean $\Delta\Delta C_T$ Mut-WT
10 ⁹	- 9.15	20 ng	- 9.19
10 ⁸	- 9.53	15 ng	- 9.07
10 ⁷	- 9.72	10 ng	- 9.13
10 ⁶	- 9.41	5 ng	- 9.09

	C_T Mean ARMS ^a	C_T Mean <i>Mt-COX3</i>	Normalized to <i>Mt-COX3</i>	ΔC_T	$\Delta\Delta C_T$
143B (WT)	28.40	13.75	25.36	8.72	
ND1 ^m	16.64	11.46	16.64		
ND1 ^m + oND1-FLAG	18.01	12.83	16.09	- 0.55	- 9.27

^a Using primers ND1Mut-F and ND1-R (Supplementary Table S3)

Supplementary Table S5

qPCR primers for mRNA quantification in HEK293

Primer	Sequence
ATP5G1 Forward	5' CCAGACCAGTGTGTCTCCC3'
rND1 Reverse	5' CTTTGCGTAGTTGCATATAGCC3'
oND1 Reverse	5' ATTGCGTCAGCGAATGGC3'
rND2 Reverse	5' GCTTCTGTGGAACGAGGGTT3'
oND2 Reverse	5' CACTGGGATAAAGGCCAGCA3'
rND3 Reverse	5' TGTAGCCGTTGAGTTGTGGT3'
oND3 Reverse	5' GGGTCGAATCCGCACTCATA3'
rND4 Reverse	5' TAGGGGGTCGGAGGAAAAGG3'
oND4 Reverse	5' GGGAGCAGCTGAACAGGTTA3'
rND4L Reverse	5' GGACATGAGGTGTGAGCGATA3'
oND4L Reverse	5' AGCTCATCAGGTGAGACCGA3'
rND5 Reverse	5' CCCAGTGCCAGTTCGAGATA 3'
oND5 Reverse	5' AAGGTAGAGGCCACGATGGA3'
rND6 Reverse (*)	5' CCGACCACACCGCTAACAAT3'
oND6 Reverse (*)	5' CCGCCGAAGTTCAGGATGAT3'
rCOX1 Reverse	5' TGCCTAGGACTCCAGCC3'
oCOX1 Reverse	5' GAACAGCAGGTACAGTGTGCC3'
rCOX2 Reverse	5' TGATAAGCTCTTCCATGATAGG3'
oCOX2 Reverse	5' TGATCAGCTCCTCCATGATG3'

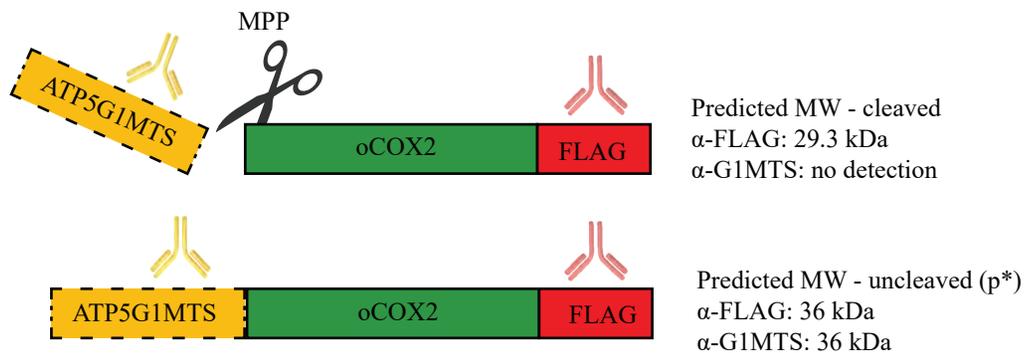
rCOX3 Reverse	5' TCATGGAGTGGAAGTGAAAC3'
oCOX3 Reverse	5' GGCCAGAGGTCATCAGCA3'
rCYB Reverse	5' CATGCGGAGATGTTGGATGG3'
oCYB Reverse	5' GAGATGTTGCTGGGTGTTGG3'
rATP6 Reverse	5' ATGATCAGTACTGCGGCGG3'
oATP6 Reverse	5' TGCAGGCAGTCCCAGAAT3'
rATP8 Reverse	5' TGAGGAATAGTGTAAGGAGCATGG3'
oATP8 Reverse	5' TCAGGAACAGTGTCAGCAGC3'
GAPDH forward	5' CCCACCCACACTGAATCTCC3'
GAPDH reverse	5' GTACATGACAAGGTGCGGCT3'
COX10 forward	5' GTTCCGGGAGTTGCCATTCTG3'
COX10 reverse	5' CAACCACAGCTCCGACCCATG3'

* Primers not used as rND6 and oND6 were not compared

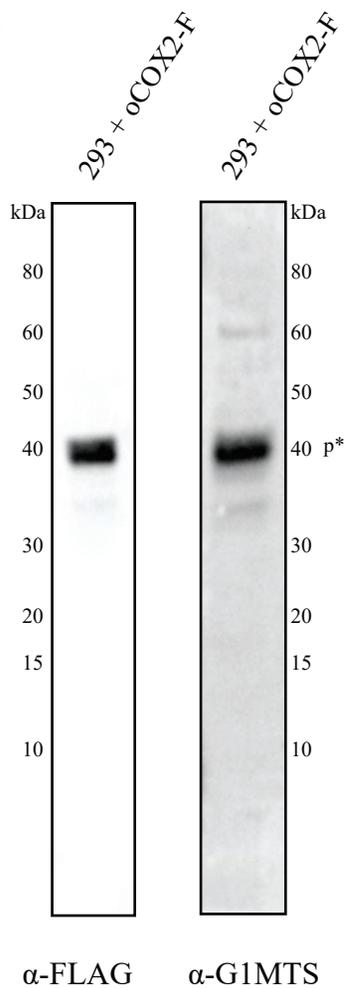
Supplementary Figure S1

S1 The MTS of oCOX2 is not cleaved

A



B

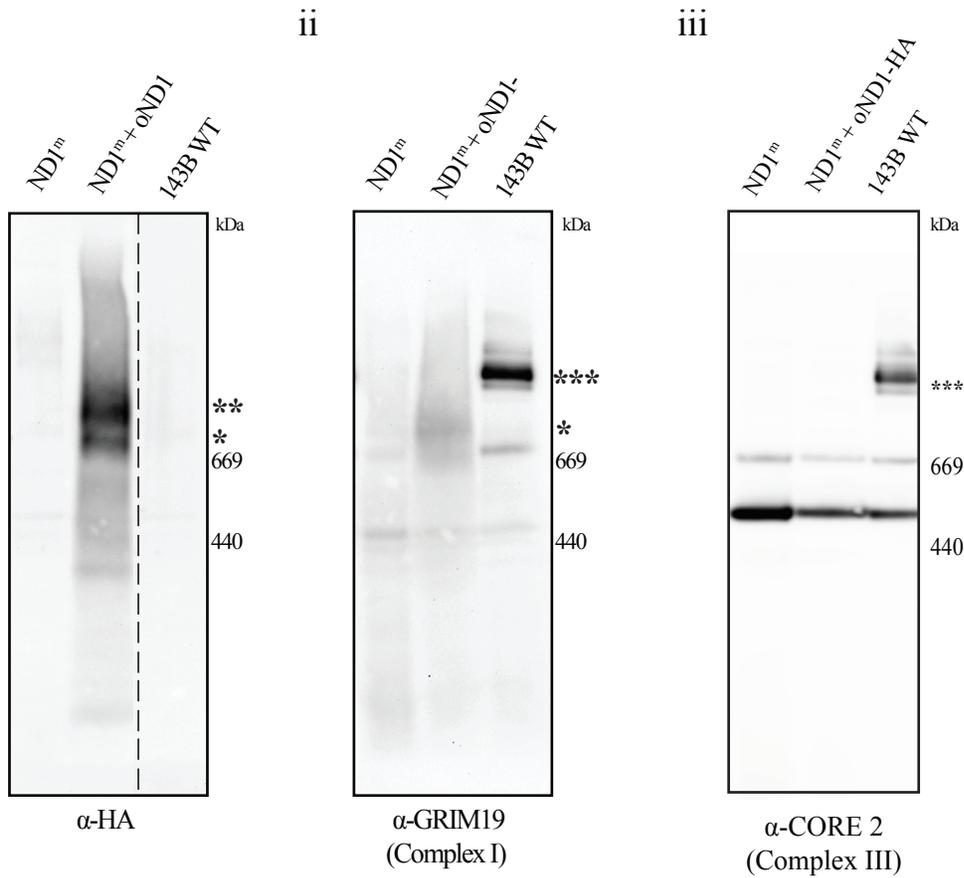


Supplementary Figure S1 The MTS of oCOX2 is not cleaved. A) Schematic showing the expected molecular weight of allotopically expressed oCOX2 when the ATP5G1 MTS is properly cleaved (top) or when it fails to be cleaved (bottom) upon mitochondrial import. B) Denaturing PAGE of mitochondria enriched fractions (~20 μ g protein/ lane) from HEK293 cells stably expressing optimized COX2 (oCOX2) probed for the FLAG epitope (left) and against the ATP5G1 MTS (right).

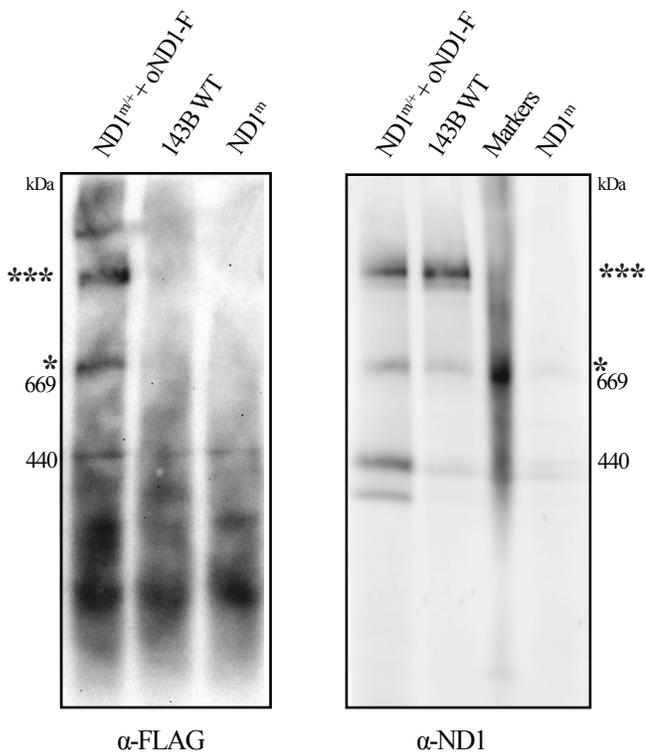
Data Information: p* predicted precursor (uncleaved) protein. Representative blot of n=3 biological replicates.

Supplementary Figure S2

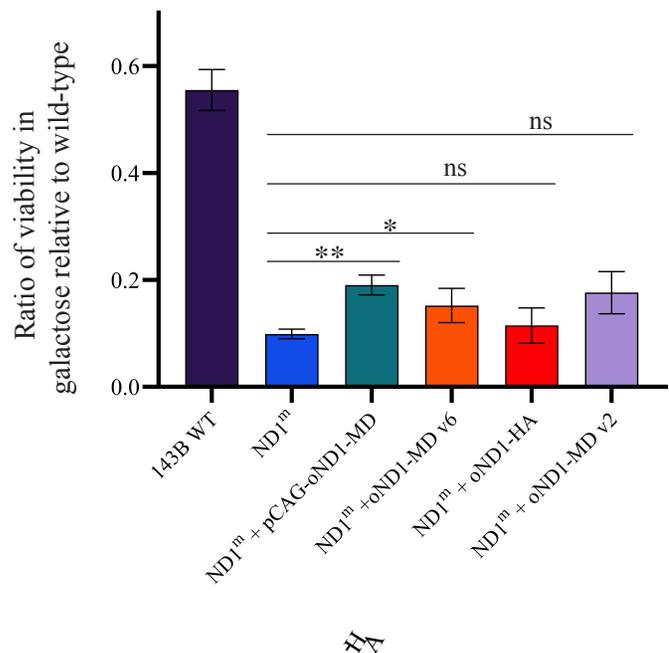
S2A HA-tagged oND1 does not restore Complex I Assembly



S2B Efficient incorporation of oND1-F into Complex I in heteroplasmic ND1^{m/+}



S2C Galactose Viability of ND1^m Cell Lines Stably Expressing oND1



Supplementary Figure S2 Rescue capacity of oND1 varies with construct design and heteroplasmy

(A) HA-tagged oND1 does not restore Complex I assembly in ND1^m. Digitonin-lysed mitochondria from WT, ND1^m, and ND1^m expressing oND1-HA run on Blue Native PAGE followed by immunoblotting. (i) Probed with HA-epitope tag and (ii) probed against CI subunit GRIM19. The CI monomer is absent in the ND1 *null* line, and is partially restored in *null* cells expressing oND1-HA (**), along with a late-stage CI intermediate (*). Mitochondria of wild-type 143B cells possess CI only in its supramolecular form (***), which co-migrates with CIII. (iii) Probed against Complex III subunit CORE II. The Complex III monomer and dimer are not perturbed in the mutant line, however the CIII/I supercomplex (***) is absent in ND1 *null* cells and in *null* cells expressing oND1-HA. Representative blots of n=3 biological replicates.

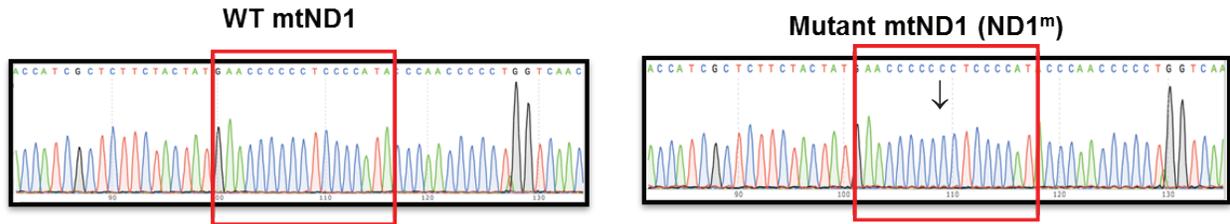
(B) Allotopically-expressed oND1-Flag incorporates into Complex I monomers and respiratory supercomplexes in heteroplasmic ND1^{m/+}. Mitochondrial lysates from heteroplasmic ND1 *null* cells expressing oND1-FLAG (ND1^{m/+} +oND1-F), WT (143B), and ND1 homoplasmic *null* (ND1^m) run on BN-PAGE followed by immunoblotting against (i) FLAG epitope and (ii) endogenous ND1 protein. Both FLAG-tagged oND1 and Native ND1 are observed in high molecular weight complex assemblies (***) and in CI monomers (**). Representative blots of n=3 biological replicates.

C) Viability on galactose media of ND1^m cells stably expressing oND1. Wild type 143B cells (WT), homoplasmic ND1^m, and homoplasmic ND1^m cells stably expressing oND1-FLAG or oND1-HA were placed in either 5 mM glucose or 5 mM galactose containing medium for 72 h and assessed for viability. Data are graphed as the ratio of viability in galactose vs in glucose for each cell line. A ratio of 1.0 represents wild-type (143B) viability in glucose. Data points for oND1-MDv2 and oND1-MDv6 indicate stable cell line biological replicates containing the FLAG-tagged oND1 construct, and the ND1^m + oND1-HA line possesses the same vector construct with a 3xHA epitope in lieu of FLAG. The pCAG-oND1-MD cell line has a CAG promoter in place of CMV. n=4 biological replicates.

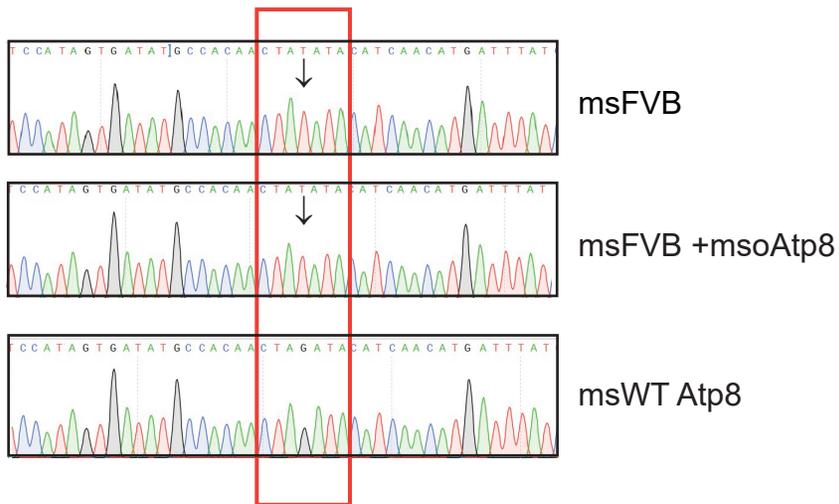
Data Information: In (A-B), *Complex I late-stage assembly intermediate **Complex I monomer ***Supercomplex with Complex I/III. In (C), data are average ratio \pm SEM. *** $P=0.0007$ [ND1^m vs pCAG-oND1] * $P=0.0126$ [ND1^m vs oND1-MDv6] ns - not significant [ND1^m vs oND1-HA $P > 0.9999$; ND1^m vs oND1-MDv2 $P = 0.0634$] (Dunnett's multiple comparison test after ANOVA, vs ND1^m).

S3

S3A Sanger Sequencing of WT or ND1^m mtDNA (m.3571insC)



S3B Sanger Sequencing of msAtp8 mutation in mouse cell lines



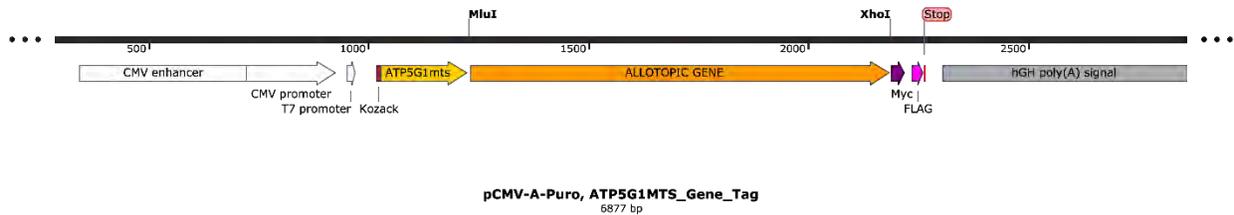
Sequencing primer MsmtDNA7611 Fwd

5' - GTG GAT CTA ACC ATA GCT TTA TGC CC- 3'

Supplementary Figure S4

Schematic of Cloning Strategy for Minimally-Recoded and Codon-Optimized Genes

All sequences were synthesized without a stop codon, and with 5' MluI and 3' XhoI restriction sites. Sub-cloning into the target vector introduced the gene downstream of the promoter and *ATP5G1* MTS and upstream of the epitope tag, followed by a stop codon.



Supplementary Data S1

Minimally-Recoded and Codon-Optimized Gene Sequences

rND1

```
ATGCCCATGGCCAACCTCCTACTCCTCATTGTACCCATTCTAATCGCAATGGCATTCCCTAATGCTTACCG
AACGAAAAATTCTAGGCTATATGCAACTACGCAAAGGCCCAACGTTGTAGGCCCTACGGGCTACTACA
ACCCTTCGCTGACGCCATGAAACTCTTCACCAAAGAGCCCCCTAAAACCCGCCACATCTACCATCACCTC
TACATCACCGCCCCGACCTTAGCTCTCACCATCGCTCTTCTACTATGGACCCCCCTCCCCATGCCAACC
CCCTGGTCAACCTCAACCTAGGCCTCCTATTTATTCTAGCCACCTCTAGCCTAGCCGTTTACTCAATCCT
CTGGTCAGGGTGGGCATCAAACCTCAAACCTACGCCCTGATCGGCGCACTGCGAGCAGTAGCCCAAACAATC
TCATATGAAGTCACCCTAGCCATCATTCTACTATCAACATTACTAATGAGTGGCTCCTTTAACCTCTCCA
CCCTTATCACAACACAAGAACACCTCTGGTTACTCCTGCCATCATGGCCCTTGCCATGATGTGGTTTTAT
CTCCACACTAGCAGAGACCAACCGAACCCCTTCGACCTTGCCGAAGGGGAGTCCGAAGTACTCTCAGGC
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rCOX2

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oCOX2

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rCOX3

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oCOX3

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rATP6

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oATP6

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rATP8

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oATP8

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Mouse rATP8

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Mouse oATP8

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* rND6 could not be synthesized